(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 667 165 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
27.03.2002 Bulletin 2002/13

(51) Int Cl.[7]: **A61K 39/395**
// (A61K39/395, 33:24), (A61K39/395, 31:70)

(21) Application number: **94117268.6**

(22) Date of filing: **15.09.1989**

(54) **Therapeutic compositions containing monoclonal antibodies specific to human epidermal growth factor receptor**

Therapeutische Zusammensetzungen die monoklonale Antikörper enthalten gegen den menschlichen Rezeptor für epidermalen Wachstumsfaktor

Compositions thérapeutiques contenant des anticorps monoclonaux contre le récepteur humain du facteur de croissance épidermique

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(30) Priority: **15.09.1988 US 244737**
03.03.1989 US 319109

(43) Date of publication of application:
16.08.1995 Bulletin 1995/33

(83) **Declaration under Rule 28(4) EPC (expert solution)**

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
89117125.8 / 0 359 282

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Description

[0001] This invention relates to therapeutic compositions containing monoclonal antibodies specific to a human receptor for epidermal growth factor (EGF) which can inhibit the growth of human tumor cells that express human EGF receptors, in combination with anti-neoplastic agents.

[0002] Control of cell growth is regulated by the interaction of soluble growth factors and cell membrane receptors.

[0003] The first step in the mitogenic stimulation of epidermal cells is the specific binding of epidermal growth factor (EGF) to a membrane glycoprotein known as the epidermal growth factor receptor (EGF receptor). (Carpenter, et al., Epidermal Growth Factor, Annual Review Biochem., Vol. 48, 193-216 (1979)). The EGF receptor is composed of 1,186 amino acids which are divided into an extracellular portion of 621 residues and a cytoplasmic portion of 542 residues connected by a single hydrophobic transmembrane segment of 23 residues. (Ullrich, et al., Human Epidermal Growth Factor cDNA Sequence and Aberrant Expression of the Amplified Gene in A-431 Epidermoid Carcinoma Cells, Nature, Vol. 309, 418-425 (1986)). The external portion of the EGF receptor can be subdivided into four domains. Recently, it has been demonstrated that domain III, residues 333 to 460, which is flanked by two cysteine domains is likely to contain the EGF binding site of the receptor. (Lax, et al., Localization of a Major Receptor-Binding Domain for Epidermal Growth Factor by Affinity Labelling, Mol. and Cell Biol., Vol. 8, 1831-1834 (1988)). The binding of EGF to domain III leads to the initiation of pleiotropic responses leading to DNA synthesis and cell proliferation.

[0004] It has been found in various types of human tumor cells that those cells overexpress EGF receptors. For example, the cancerous cells of bladder tumors have been shown to have a relatively large population of EGF receptors. (Neal, et al., Epidermal Growth Factor Receptor in Human Bladder Cancer: Comparison of Invasive and Superficial Tumors, Lancet, Vol. 1, 366-367 (1985)). Breast cancer cells exhibit a positive correlation between EGF receptor density and tumor size and a negative correlation with the extent of differentiation. (Sainsbury, et al., Epidermal Growth Factor Receptors and Ostrogen Receptors in Human Breast Cancer, Lancet, Vol. 1, 364-366 (1985); Presence of Epidermal Growth Factor Receptor as an Indicator of Poor Prognosis in Patients with Breast Cancer, J. Clin. Path., Vol. 38, 1225-1228; Epidermal-Growth-Factor Receptor Status as Predictor of Early Recurrence and Death From Breast Cancer, Lancet, Vol. 1, 1398-1400 (1987)). The tumorigenicity of a series of human vulval epidermoid carcinoma (A431) clonal variants implanted into athymic mice having different levels of EGF receptors was found to correlate directly with the level of expression of the EGF receptor (Santon, et al., Effects of Epidermal Growth Factor Receptor Concentration on Tumorigenicity of A431 Cells in Nude Mice, Cancer Res., Vol. 46, 4701-4700 (1986)). Thus, it has been proposed that overexpression of EGF receptors play a role in the origin of tumorigenesis of cancer cells.

[0005] The influence of EGF receptor density on the biological behavior of cancer cells may be mediated by the interaction of the receptor with its ligands - namely, EGF or transforming growth factor (TGF). In the majority of cells, when EGF binds to a specific region of the EGF receptor, the cell is mitogenically stimulated. Other tumor cells, such as A431 cells, are not mitogenically stimulated by the binding of EGF to its receptors.

[0006] Two groups have reported in vivo growth inhibition of tumor A431 cell xenografts in nude mice by binding monoclonal antibodies to the epidermal growth factor receptor of the tumorous cells. Masui, et al. demonstrated that treatment with anti-EGF receptor monoclonal antibodies of the IgG2a and IgGl isotype completely prevented tumor formation in athymic mice by sub-cutaneously implanted A431 cells when treatment was started on the day of tumor cell inoculation. (Masui, et al., Growth Inhibition of Human Tumor Cells in Athymic Mice by Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies, Cancer Res., Vol. 44, 1002-1007 (1984); Mechanism of Antitumor Activity in Mice for Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies With Different Isotypes, Cancer Res., Vol. 46, 5592-5598 (1986)). Rodeck, et al. used a different monoclonal antibody than Masui of the.IgG2a isotype which also binds to the EGF receptor of A431 cells to completely inhibit tumor growth of A431 cells xenotransplanted in mice. (Rodeck, et al., Tumor Growth Modulation by a Monoclonal Antibody to the Epidermal Growth Factor Receptor: Immunologically Mediated and Effector Cell - Independent Effects, Cancer Res., Vol. 47, 3692-3696 (1987)).

[0007] To date, no one, however, has inhibited the in vitro or in vivo growth of human oral epidermoid carcinoma (KB) or human mammary epithelial (184A1N4 and 184A1N4-T-collectively "184") cells. KB and 184 cells are commonly used in studies relating to the EGF-receptor.

[0008] KB and 184 cells are substantially different from A431 cells, especially in terms of their growth response to epidermal growth factor. KB and 184 cells are growth stimulated by high concentrations of epidermal growth factor whereas A431 cells are growth inhibited by high concentrations of epidermal growth factor.

[0009] Those differences as well as the lack of complete understanding of the mechanism by which the anti-EGF-receptor antibodies inhibit the growth of tumor cells in vivo, prohibit one from accurately determining whether monoclonal antibodies which bind to EGF receptor of A431 cells and demonstrate antitumoral activity on A431 cell xenografts in nude mice will also demonstrate antitumoral activity on KB or 184 cell xenografts in nude mice.

[0010] Additionally, because human tumor cells are

also growth stimulated by epidermal growth factor, KB and 184 cells provide a more representative pattern of responding to EGF than A431 cells, and, in fact, are used as a model for human tumor cells expressing EGF receptors. (Willington, et al. J. Cell Biol., Vol. 94, 207-212 (1982)).

[0011] The primary goal in treating tumors is to kill all the cells of the tumor. A therapeutic agent that kills the cell is defined as cytotoxic. A therapeutic agent that merely prevents the cells from replicating, rather. than killing the cells, is defined as cytostatic.

[0012] Treatment solely with monoclonal antibodies which bind to the EGF receptor merely prevent the cells from replicating, and thus, the monoclonal antibodies act as a cytostatic agent. In order to overcome the monoclonal antibody's cytostatic limitations, monoclonal antibodies specific to the extracellular domain of human epidermal growth factor receptors have been combined with macrophage or mouse complement to yield a cytotoxic response against A431 cells. (Masiu, et al., Mechanism of Antitumor Activity in Mice for Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies with Different Isotopes, Cancer Research, Vol. 46, 5592-5598 (1986)).

[0013] Anti-neoplastic or chemotherapeutic agents administered by themselves, are effective cytotoxic agents. The use of anti-neoplastic agents such as doxorubicin (adriamycin) and cisplatin, for example, are well known in the art. Use of those reagents by themselves, however, are only effective at levels which are toxic or subtoxic to the patient. Cisplatin is intravenously administered as a 100 mg/m^2 dose once every four weeks and adriamycin is intravenously administered as a 60-75 mg/m^2 dose once every 21 days.

[0014] In the present invention monoclonal antibodies are used which inhibit the growth of human tumor cells by binding to the extra-cellular domain of the human EGF receptors of said tumor cells in an antigen-antibody complex, with the tumor cells characterized by their expression of human EGF receptors and by mitogenic stimulation by human EGF.

[0015] The present invention provides a therapeutic composition comprising:

(a) a monoclonal antibody which inhibits the growth of human tumor cells by said antibody binding to the extra-cellular domain of the human EGF receptors of said tumor cells in an antigen-antibody complex, said tumor cells being characterized by their expression of human EGF receptors and mitogenic stimulation by human EGF and

(b) an anti-neoplastic agent,

wherein the antibody is not antibody 108 produced by hybridoma cell line ATCC HB 9764 or antibody 96 produced by hybridoma cell line ATCC HB 9763.

[0016] The method of producing the monoclonal antibody which binds to the extracellular domain of a human EGF receptor and which is capable of inhibiting the growth of human cancer cells that express human EGF receptors and are mitogenically stimulated by EGF comprises the steps of: (i) immunizing mice with a cell expressing human EGF receptor; (ii) removing the spleens from said mice and making a suspension of the spleen cells; (iii) fusing said spleen cells with mouse myeloma cells in the presence of a fusion promoter; (iv) diluting and culturing the fused cells in separate wells in a medium which will not support the unfused myeloma cells; (v) evaluating the supernatant in each well containing a hybridoma for the presence of antibody to human EGF receptor; (vi) selecting and cloning a hybridoma producing antibody which binds to the extra-cellular domain of a human EGF receptor; and (vii) recovering the antibody from the supernatant above said clones.

[0017] A further method of producing the monoclonal antibody omits step (vii) described above and contains the further steps: (viii) transferring said clones intraperitoneally into mice; and (ix) harvesting the malignant ascites or serum from said mice, which ascites or serum contains the desired antibody.

[0018] Applicant has surprisingly discovered that the combined treatment of one of the novel monoclonal antibodies with anti-neoplastic drugs such as doxorubicin or cisplatin provides a more efficient treatment for inhibiting the growth of human cancer cells that express human EGF receptors and are mitogenically stimulated by human EGF than the use of the novel monoclonal antibody of the anti-neoplastic agent by itself. The combined treatment using the novel monoclonal antibodies is advantageous because it combines two anti-cancer agents, each operating via a different mechanism of action to yield a cytotoxic effect to human tumor cells. That approach could solve problems arising in the clinic, such as, on the one hand, the development of resistance to drugs, and on the other hand, a change in the antigenicity of the tumor cells that would render them unreactive with the antibody. Furthermore, applicant has also surprisingly discovered that the anti-neoplastic agent can be administered at levels substantially lower than the levels required when administering the anti-neoplastic agent by itself, which are toxic or subtoxic to the patient. Anti-neoplastic agents other than doxorubicin or cisplatin such as bleomycin sulfate, carmustine, chlorambucil, and cyclophosphamide hydroxyurea may also be used with the novel monoclonal antibody. The aforementioned list is merely exemplary and is not intended to limit the scope of the invention.

[0019] This invention also provides a method for inhibiting the growth of human tumor cells that express human EGF receptors and are mitogenically stimulated by human EGF which comprises administering an effective amount of an anti-neoplastic agent and an effective amount of either one of the novel monoclonal antibodies to a human cancer patient having said tumor cells, whereby the antibody binds to the extra-cellular domain

of the human EGF receptor of the tumor cell in an antigen-antibody complex.

[0020] A more complete appreciation of the present invention and many of the attendant advantages thereof will be readily obtained as the invention becomes better understood by reference to the following detailed description in connection with the accompanying drawings. Descriptions of embodiments no longer covered by the claims of the present invention are maintained as examples for the methods used in the present invention.

[0021] Figure 1 demonstrates a sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the $F(ab)'_2$ and F(ab) preparations of 108 monoclonal antibody. The gel was run under non-reducing conditions a) intact 108 monoclonal antibody, b) unpurified $F(ab)'_2$, fragment preparation, c) purified $F(ab)'_2$, d) Fab fragment, e) Molecular weight markers, KD.

[0022] Figure 2 demonstrates competitive binding of ^{125}I 108 monoclonal antibody and its fragments with EGF to KB cells. KB cells were incubated in the presence of $3x10^{-9}M$ ^{125}I 108 monoclonal antibody ($1x10^6$ cpm/ml) in the presence of different concentrations of EGF ((●), unlabelled 108 monoclonal antibody (○), its $F(ab)'_2$, fragment (▲) or Fab' fragment (■). (Average of 3 independent measurements).

[0023] Figure 3 demonstrates a cell sorter analysis of the binding of 108 monoclonal antibody to KB cells.

[0024] Figure 4 demonstrates the homing of ^{125}I 108 monoclonal antibody to KB cells implanted in nude mice.

[0025] Figure 5 demonstrates the effect of EGF and 108 monoclonal antibody on colony formation of KB cells. Colony formation assay was carried out at described in Materials and methods in the presence of different concentrations of EGF (●). 108 monoclonal antibody (■).

[0026] Figure 6 demonstrates the antitumor activity of 108 monoclonal antibody and its fragments against KB cells implanted in nude mice. Each group contained at least six mice. Mice were treated intravenously at days 1, 5, 12, and 18 after tumor inoculation with 1 mg of 108 monoclonal antibody (■), 1 mg monoclonal antibody to DNP (○), 0.66 mg $F(ab)'_2$, (▲) or Fab' (♦) fragments of 108 monoclonal antibody. A single 2 mg 108 monoclonal antibody treatment one day after tumor cells injection (●).

[0027] Figure 7 demonstrates antitumor activity of 108 mAb against KB cells implanted intraperitoneally in nude mice. Seven mice were treated intravenously at days 1, 4, and 7 after tumor inoculation with 0.5 mg of 108 monoclonal antibody (---) or antibody to DNP (—) (8 mice).

[0028] Figure 8 demonstrates antitumor activity of 108 mAb against KB cells injected intravenously to nude mice. a) Histology of a lung six weeks after intravenous injection of 1.5×10^6 KB cells showing micrometastasis; x 250. b) Mice were treated intravenously with 0.5 mg of 108 monoclonal antibody at days 6, 9, and 13 after tumor inoculation. Each point represents analysis of serial sections taken at different depths through the lungs of animals.

[0029] Figure 9 demonstrates antitumor activity of 108 mAb in combination with doxorubicin against KB cells implanted subcutaneously. Four doses of .45 mg of 108 monoclonal antibody of 37.5 ug of doxorubicin were given 24 hours after the tumor injection and repeated 3 times at 3-4 day intervals.

[0030] Figures 10 and 11 demonstrate antitumor activity of 108 mAb in combination with cisplatin against KB cells implanted subcutaneously. In Figure 10 one treatment comprising 1.8 mg 108 monoclonal antibody and 100 ug cisplatin was administered. In Figure 11 mice were treated intravenously a single time 20 hours after the tumor implantation with 19 mg of 108 monoclonal antibody and 0.1 mg cisplatin (Abic, Ramat-Gan, Israel). Each of the substances were separately injected, PBS (●), monoclonal antibody (▲), cisplatin (■), and monoclonal antibody + cisplatin (♦).

[0031] Figure 12 demonstrates the effect of EGF on cell growth. A) 184A1N4 cells. B) MDA-468 cells. 184A1N4 cells were passed (5,000/well) into triplicate wells of 24-well plates and EGF was added. MDA-468 cells were passed (5,000/well) into triplicate wells of 24-well plates and allowed to attach overnight. EGF was added the following day. Media was changed after 48 h and the cells were counted after 4 days. Data are mean (±SD) cell numbers.

[0032] Figure 13 demonstrates anti-EGF receptor antibody (aEGPR) inhibition of anchorage dependent cell growth. 184A1N4 (A and B) and MDA-468 (C and D) cells were passed (5,000/well) into triplicate wells of 24-well plates and allowed to attach before antibody was added. 184A1N4 growth media contained 1 ng/ml EGF. Growth media was changed after 48 h and the cells were counted after 4 days. Data is % control cell numbers (mean±SD). 96 IgM (●), 42 IgM(○), nonspecific IgM(Δ), 225 IgG(■), 108 IgG(□), non-specific IgG (▲).

[0033] Figure 14 demonstrates reversal of aEGFR inhibition of anchorage dependent cell growth by EGF. Cells were passed (5,000/well) into triplicate wells of 24-well plates. 184A1N4 cells (A and B) were allowed to attach for 4 h in medium containing no EGF before the addition of EGF and antibodies. MDA-468 cells (C and D) were allowed to attach overnight. Antibodies were added for a final concentration of 20nM. Media was changed after 48 h and cells were counted after 4 days. Data are mean (±SD) cell numbers. 96 IgM(●), 42.IgM (○), non-specific IgM(Δ), 225 IgG(■), 108 IgG(□), non-specific IgG(Δ).

[0034] Figure 15 demonstrates inhibition of 184A1N4-T colony formation by monoclonal aEGFR. Cells were grown in soft agar as described in Example VIII(B) in the presence of 20nM aEGFR or 20nM non-specific antibodies and increasing concentrations of EGF. Data are mean (±SD) number of colonies greater than 60 um in size. A) IgG:225IgG(●), 108 IgG (○), non-

specific IgG(Δ). B) IgM: 96 IgM (○), 42 IgM (●), non-specific IgM(Δ).

[0035] Figure 16 demonstrates the effects of aEGFR on MDA-468 colony formation. Cells were grown in soft agar as described in Example VIII(C) in the presence of 20 nM aEGFR or non-specific antibody and increasing concentrations of EGF. Cells were also grown in the presence of EGF alone. Data are mean (±SD) number of colonies greater than 60 um in size. A) IgG: 225 IgG (●), 108 IgG(Δ), non-specific IgG(▲), EGF alone (○). B) IgM: 96 IgM(Δ), 42 IgM(●), non-specific IgM(▲) EGF alone (○).

[0036] Figure 17 demonstrates the effects of aEGFR on [^{125}I]EGF binding to MDA-468 cells. Confluent MDA-468 cells in 24-well plates were incubated at 4°C for 2.5 h with iodinated EGF (1 nM) and increasing concentrations of unlabeled antibody or EGF. Data are the means (±SD) of duplicate determinations from 2 or 3 separate experiments. A) 225 IgG(Δ), 108 IgG(◊), non-specific IgG(▽) EGF standard(●). B) IgM: 96 IgM(●), 42 IgM(Δ), non-specific IgM(▼), EGF standard(○).

EXAMPLE I

Production of Monoclonal Antibodies

A. Immunization and Somatic Cell Hybridization

[0037] Balb/c mice were immunized by intraperitioneal injections of CH 71 cells or CH 71 cell membrane preparation. CH 71 cells are chinese hamster ovary cells which have been transfected with a plasmid bearing a truncated form (deletion of most of the intracellular domain of the EGF-R) of the EGF-R cDNA (Livneh, et al., J. Biol. Chem., Vol. 260, 12490 (1986)). These transfected cells express approximately 10^6 mutant EGF-R molecules/cell. The choice of CH-71 cells allows the selection in the first screening test of only hybridomas secreting antibodies against the extracellular domain of the EGF-R and avoids the selection of antibodies directed against the human specific carbohydrates linked to the human EGF-R molecule.

[0038] The mice were immunized three times on day 0, 13, and 32. The two best responding mice were each boosted by three intraperitioneal injections of CH 71 cells three consecutive days before the fusion. On day 65, the spleen cells of the mice were then fused with NSI myeloma cells (ratio 5/1) according to the general procedure of Kohler and Milstein, using PEG 4000 (Merck) as the fusing agent. (Kohler and Milstein, Eur. J. Immun., Vol. 6, 511-519, 1976).

B. Selection and Growth of Hybridoma

[0039] The fusion product was diluted in hypoxanthine-azaserine (HA) selection medium (G. Buttin, et al., Current Topics in Microbiology and Immunology, Vol. 81, 27-36, (1978)) instead of the hypoxanthine-aminopterin-thymidine (HAT) selection medium and distributed in 96 well plates.

[0040] The presence of specific antibodies in the medium of the wells of the growing hybridoma cells was first assayed by radioimmunoassay. Cells expressing or not expressing the EGF receptor were plated in 96 well plates. At confluency, they were washed once with binding medium (DMEM, 20 mM Hepes, 0.2% BSA) and incubated for 90 min at room temperature with 100 ul of culture supernatant from the different growing hybridomas. Cells were then washed 3 times with binding medium and incubated for a further 60 min at room temperature with 100 ul of a solution of iodinated goat anti-mouse immunoglobulins (250,000 cpm/100 ul). After 3 washes with PBS (phosphate buffered saline, pH 7.5) the cells were scraped from the wells and the radioactivity which was associated with their surface was counted using a gamma counter. The ability of the antibodies to bind specifically to the surface of cells expressing the EGF receptor (A 431, human fibroblasts or mouse 3T3 cells transfected with human EGF-R DNA constructs) was measured in this way and compared to their ability to bind to cells that do not express the EGF-R (a particular clone of mouse 3T3 cells). The positive hybridomas were cloned by limiting dilution and further tested by measuring their ability to immunoprecipitate ^{35}S methionine or ^{32}P labeled EGF-R from lysates of cell lines of different species (human, mouse, chicken). For this, goat antimouse immunoglobulins were bound to Protein A-Sepharose by incubation of goat antimouse antibody solution with Protein A-Sepharose beads for 30 min at room temperature. This was followed by washing 3 times with 20 mM Hepes, pH 7.4. Then the goat mouse Ig's coated Protein A-Sepharose beads were further incubated for 30 min at room temperature with the culture supernatant of the hybridomas, washed 3 times with HNTG buffer (20 mM Hepes , 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and incubated for 1 hour at 4 degrees C with the different cell lysates obtained by lysing cell monolayers with solubilization buffer (1% Triton X-100, 150mM NaCl, 20 mM Hepes, 1.5 mM EGTA, 1.5 mM Mg Cl_2, 10% glycerol, aprotinin, leupeptin and PMSF as protease inhibitors) and centrifugation of the lysate to discard the nuclear pellet. For ^{32}P labelling, the immunoprecipitates were washed with HNTG 3 times and then incubated for 15 min with a ^{32}P ATP solution (HNTG with 5 mM $MnCl_2$ and 3 uCi/sample of ^{32}P ATP). Electrophoresis sample buffer was then added and the samples boiled for 10 min at 95 degrees C prior loading on a 7.5% SDS-polyacrylamide gel. Monoclonal antibodies 108, 96 and 42 were all found to be specific for the human EGF-R. These antibodies were also tested for their ability to inhibit the binding of iodinated EGF to the surface of cells expressing EGF-R. These 3 antibodies inhibit the binding of EGF to its receptor, but the level of inhibition varied with 96 > 108 > 42.

EXAMPLE II

Culturing of Cell Lines

A. Culturing of Human Oral Epidermoid Carcinoma Cells (KB Cells)

[0041] The KB tumor cell line derived from oral epidermoid carcinoma was obtained from the American Type Tissue Culture Collection. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum depleted of complement activity by incubation at 56°C for 30 min and grown in glutamine, penicillin, streptomycin and sodium pyruvate, at 37°C in 5% CO_2: 95% air atmosphere.

B. Culturing of Human Mammary Epithelial Cells (184 cells) and Human Breast Cancer Cells (MDA-468 cells)

[0042] 184A1N4 and 184A1N4-T human mammary epithelial cells were provided by Martha Stampfer, Lawrence Berkeley Laboratory, Berkeley, CA. 184A1N4 cells were maintained at 37°C in 5% CO_2 and IMEM supplemented with glutamine (0.6 mg/ml), fetal calf serum (0.5%), hydrocortisone (0.5 ug/ml), insulin (5 ug/ml) and EGF (10 ng/ml). 184A1N4-T were maintained at 37°C in 5% CO_2 in IMEM (Biofluids, Rockville, MD) supplemented with glutamine (0.6 mg/ml), gentamicin (40 mg/ml) and 10% fetal calf serum. MDA-468 cells were cultured under the same conditions and medium as 184A1N4-T cells.

C. Culturing of 96 IgM and 108 IgG2a Hybridoma Cell Lines

[0043] The 108 IgG2a hybridoma cell line was generated by immunizing mice with CH 71 cells expressing the EGF receptor and cultured under the same conditions as the KB cell line. The 96 IgM hybridoma cell line was generated by the same procedure as that described for the 108 IgG2a hybridoma cell line.

EXAMPLE III

A. Purification of 108 Monoclonal Antibodies from Animals

[0044] Ascites from animals injected with the 108 IgG2a hybridoma cells were clarified by centrifugation in an eppendorf centrifuge at 4°C for 10 min. Monoclonal antibodies were precipitated by slow addition of saturated ammonium sulfate at 4°C to a final concentration of 45% (v/v), pH 7.5, for 24 hours. The precipitate was collected by centrifugation at 10,000 g for 15 minutes and washed twice with 50% v/v ammonium sulfate, pH 7.5. at 4°C. Further purification was carried out by affinity chromatography on Sepharose CL Protein A (Pharmacia) in 0.14 M Tris buffer, pH 8.0 and the 108 monoclonal antibody was eluted with 0.1 M citrate buffer, pH 3.0, followed by extensive dialysis against PBS.

B. Purification of 96 Monoclonal Antibodies from Animals

[0045] Ascites from animals injected with the 96 IgM hybridoma cells were clarified by centrifugation in a low speed centrifuge at 3000 RPM for 15 min, at 4°C. Monoclonal antibodies were precipitated by slow addition of saturated ammonium sulfate at 4°C to a final concentration of 45% (v/v), pH 7.5, for 24 hours. The precipitate was collected by centrifugation at 10,000 g for 15 minutes and washed twice with 50% v/v ammonium sulfate, pH 7.5 at 4°C. The precipitate was then dissolved in and dialyzed extensively against 50 mM Tris pH 8, 0.5 M NaCl. This material was semi-purified by gel filtration using Sephacryl S-3000 equilibrated in 50 mM Tris, pH 7.8, 0.5 M NaCl. The peak containing the mAb96 antibody was pooled and dialyzed against PBS.

EXAMPLE IV

Purification, Specific Activity and Immunoreactivity of F(ab)'2 and F(ab)' Fragment of 108 Monoclonal Antibody

[0046] 108 monoclonal antibody (5 mg/ml) in 0.1 M sodium-acetate buffer at pH 3.9 was digested in the presence of 4% w/w pepsin (Worthington Biochemical Corporation, New Jersey) for 7 h at 37°C. Digestion was terminated by adjusting the pH to 8.0 with 2 M Tris, followed by dialysis against PBS at 4°C. Remaining intact IgG molecules were removed by Protein A affinity chromatography. The Fc portion and smaller fragments were removed by gel filtration on Sepharose G-100. For the preparation of monovalent Fab' fragment, the $F(ab)'_2$ (2 mg/ml) was reduced by 10 mM dithiothreitol in 20 mM Tris buffer, pH 8.2, for 1 h at 37°C. Alkylation was performed in 40 mM iodoacetamide for 30 min at 37°C, followed by extensive dialysis against PBS at 4°C. Purity and complete digestion of the various fragments were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, see Figure 1). ^{125}I-labeling of 108 monoclonal antibody was performed by the chloramine T method (Hunter and Greenwood, Preparation of ^{131}Iodine Labelled Human Growth Hormone of High Specific Activity, Nature, Vol. 196, 465-6, (1962)). Specific activities of about $3X10^6$ cpm/ug IgG were usually obtained.

[0047] The $F(ab)'_2$ and F(ab) fragments of 108 monoclonal antibody were fully immunoreactive when compared to native intact 108 monoclonal antibody in their capacity to compete with the binding of ^{125}-I labeled 108 to EGF receptors exposed on KB cells (See Figure 2).

EXAMPLE V

108 Monoclonal Antibody Binding Properties

A. 108 Monoclonal Antibody Binding Activity to Cell Surface EGF Receptors

[0048] The antibody binding activity of 108 hybridoma supernatant was determined by an indirect immunofluorescence assay. KB cells ($2X10^6$ per sample) were trypsinized 24 hours before the assay and placed in test tubes (Falcon, polystyrene round bottom tubes). Prior to assay, the KB cell suspensions were washed with cold PBS and incubated with 108 hybridoma supernatant for 45 min at 4°C. After washing with PBS containing 1% bovine serum albumin, the cells were incubated with fluorescein-labeled rabbit anti-mouse IgG for 45 min at 4°C. Cell samples were suspended in PBS and analyzed by a fluorescence cell sorter (FACS II, Bectin Dickenson, Mountainview, Ca, USA). (See Figure 3.)

[0049] Uniformity of receptor expression was shown by positive stain in at least 96% of the cells compared with absence of staining observed with supernatant of hybridoma raised against human hepatitis B virus (7H01). Scatchard analysis of antibody binding parameters at 4°C revealed an average of $2X10^5$ binding sites per cell with KD of 1.8×10^{-9} M^{-1}.

B. A Competitive Radioimmunoassay of Epidermal Growth Factor with 108 Monoclonal Antibody and its Fragments

[0050] KB cells (10^5/well in 24 well plates; NUNC) were grown for 24 hours, washed with PBS and incubated with different concentrations of either native antibody or its fragments in DMEM containing 1% bovine serum albumin for 1 h at 4°C, or at room temperature, in the presence of ^{125}I 108 monoclonal antibody (about $1X10^6$ cpm/ml). The cells were then washed, solubilized in 0.5 N NaOH and their radioactivity was determined in a counter (Kontron, Switzerland). Non-specific binding was determined by the addition of 100-fold excess of unlabelled monoclonal antibody. Results are presented as the percentage of radioactivity associated with the cells incubated with unlabelled antibody (intact or fragmented) vs. radioactivity associated with cells incubated without the addition of cold antibody.

[0051] EGF competes with the binding of the antibody to the receptor to a maximal level of about 70% (See Figure 2).

C. In Vivo Localization of the Radiolabeled 108 Monoclonal Antibody

[0052] KB cells (4×10^6) were inoculated subcutaneously on the back of nude mice (5-6 weeks old) . After 14 days, when the tumor reached a diameter of about 1.2 cm, ^{125}I 108 monoclonal antibody was injected intravenously or intraperitoneally ($5X10^6$ cpm; $3X10^6$ cpm/ug). ^{125}I-7H01 monoclonal antibody to human hepatitis B virus IgG2a served as control. Four days after the administration of antibodies, animals were killed and the radioactivity in the different tissues was determined. Means of at least four animals per group are presented. (See Figure 4)

[0053] Both intravenous and intraperitioneal administration of the tagged 108 monoclonal antibody resulted in antibody concentration at the tumor mass. Administration of control IgG resulted in no concentration at the tumor mass when given intravenously, while a marginal concentration in the tumor was detected when the antibodies were administered intraperitoneally. The percentage of injected dose accumulated at the tumor mass 96 h post intravenal injection were 7.8 ± 1.1 and $0.8. \pm 0.1$ for monoclonal antibody 108 and 7H01 monoclonal antibody (control antibody) respectively, and for the intraperitioneal injection 7.5 ± 0.4 and 1.8 ± 0.2 respectively.

EXAMPLE VI

96 Monoclonal Antibody Binding Properties

A. A Competitive Radioimmunoassay of Epidermal Growth Factor with 96 Monoclonal Antibody

[0054] Washed, confluent MDA-468 cell monolayers in 24-well culture plates were incubated at 4°C for 2.5 h with or without various concentrations of antibody or unlabeled EGF in binding buffer (IMEM, 0.1% BSA, 50 mM Hepes) [^{125}I]EGF (S.A. 80-160 uCi/ug, ICN Radiochemicals, CA) was added for a final concentration of 1 nM. After incubation the monolayers were washed, solubilized with lysis buffer (10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.4) and radioactivity was determined using a gamma-counter (LKB-Pharmacia).

[0055] All four antibodies were able to inhibit the binding of labeled EGF whereas nonspecific IgG or IgM were ineffective. The two antibodies most effective in inhibiting cell growth (125 IgM and 225 IgG) were also the most effective in inhibiting [^{125}I]EGF binding. These antibodies were able to block [^{125}I]EGF binding to a greater extent than unlabeled EGF (see Figure 17).

EXAMPLE VII

Utility of 108 Monoclonal Antibody

A. Colony Inhibition Assay of KB Cells

[0056] KB cells were seeded in petri dishes (50 x 15 nM^2, NUNC) at a concentration of 2×10^2 cells per dish. After 16 to 24 h medium was replaced with a fresh one containing different concentrations of either native or fragmented 108 monoclonal antibody with or without EGF. On the sixth day cultures were refed by fresh me-

dium containing the above ingredients. On the 15th day the cultures were washed with PBS, fixed with 4% v/v formaldehyde in PBS for 15 min and stained with hematoxylin. Number of formed colonies (25 cells) was then determined.

[0057] Figure 4 shows the effect of increasing concentrations of EGF and 108 monoclonal antibody on the growth of KB cells. Exposure of KB cells to EGF (160 nM) resulted in an increase to 150% in the number of colonies counted 15 days after seeding (14 days after the beginning of the treatment) as compared to cells incubated in the absence of growth factor. In addition EGF caused an increase in the size of KB cell colonies. When a similar experiment was performed in the presence of 108 monoclonal antibody (1.6 uM) the number of cell colonies was reduced to 30% of control values. Moreover, a 100-fold excess of 108 monoclonal antibody added together with EGF given at concentration which caused a 50% increase in the colony number, reduced the number of colonies to 20% of control values. Under the same conditions, $F(ab)'_2$ fragments of 108 monoclonal antibody had no effect on the number of KB colonies. Yet when added in 100-fold excess to EGF, the $F(ab)'_2$ fragments are able to abolish the effect of EGF on the number of formed colonies (from 150% to 103%). Incubation with the same concentration of monoclonal antibody to dinitrophenyl (DNP) did not affect the number of formed colonies. (See Figure 5).

B. Antitumoral Activity of 108 Monoclonal Antibody and its Fragments in Nude Mice

[0058] KB cells ($2X10^6$) were injected subcutaneously into nude mice, followed by either one or several intravenal injections of the 108 monoclonal antibody, starting one day after tumor cell injection. Tumor parameters were measured twice a week with a caliper and its volume was calculated according to the formula: Tumor volume (nM^3) = length x width x height. In order to validate volume measurements, correlation between tumor volume and tumor weight at the day of animal killing was assessed.

[0059] The antibody was assayed for its capacity to inhibit the growth of KB cells in nude mice (See Figure 6). Animals received 1 mg of either 108 monoclonal antibody or control monoclonal antibody to dinitrophenyl at days 1, 5, 12 and 18 after tumor inoculation. The fragments $F(ab)'_2$ and Fab' were given at antibody equivalent doses. The 108 monoclonal antibody-treated group significantly retarded tumor development and growth when compared to the group treated with control monoclonal antibody ($P<0017$, student-t test). The $F(ab)'_2$ was found to affect tumor growth but less efficiently than the whole antibody ($P<0.05$ student-t test for days 12, 17, 22, 25). Fab' fragment did not affect the tumor growth. A single 2 mg does of 108 native monoclonal antibody given one day after injection of tumor cells was found to be as efficient as four treatments of 1 mg given at days 1, 5, 12 and 18 after tumor inoculation. In another experiment, when animals were treated with a single dose of 0.66 mg $F(ab)'_2$ fragments, the antitumoral effect was slightly lower, yet a significant difference between the control and the treated group was found using the Mann Whitney analysis ($p<0.03$ for days 9, 12, 14, 17) and student-t test ($p < 0.05$ days 9, 12). At the day of sacrifice, tumors were measured and then removed for weight determination. the correlation coefficient between the tumor volume and the tumor weight was 0.95 ($P<0.0001$).

C. Tumor Growth in the Peritoneal Cavity

[0060] The injection of 3×10^6 KB cells intraperitoneally one week after mice (Nude in general background) received x-irradiation (400 rads), brought about the development of an ascitic growth. The intraperitioneal tumor-bearing mice died after 30 days. Three intravenous injections of 108 monoclonal antibody (.5 mg each) prolonged the life span of animals with 30% of animals not developing tumors at all. (See Figure 7).

D. Tumor Growth in a Metastatic Form

[0061] The metastatic form of the KB tumor could be obtained by the injection of the cells intravenously (i.v.). Mice injected with $1.5x10^6$ KB cells developed tumor nodules in the lungs 4-6 weeks after their implantation. This tumor model mimics the situation in the clinic, where tumor cells infiltrate into internal organs. This is the major problem in the treatment of cancer. The KB cell injection was followed by 3 intravenous injections of .5 mg 108 monoclonal antibody at days 6, 9, and 13 after the tumor cell injection. At the termination of the experiment, the lungs were removed, fixed in 4% formaldehyde and paraffin embedded. Serial sections were cut 4-5 um in thickness and stained with hematoxylin. The number of metastatic nodules of various depths through the lungs was obtained by light microscopy analysis. Isolation of three metastatic cell clones from lungs of tumor bearing animals and their assay for receptor levels revealed persistence of receptor expression. Treatment by the antibody reduced the number of lung tumor nodules to 15% of those in the respective controls. ($P<0.05$ Mann-Whitney analysis) (See Figure 8).

EXAMPLE VIII

Utility of 96 Monoclonal Antibody

A. 96 Inhibits 184A1N4 and MDA-468 Cell Growth

[0062] 184A1N4 and MDA-468 cells were passed (5,000/well) into triplicate wells of 24-well plates and allowed to attach before antibody was added. 184A1N4 growth media contained 1 ng/ml EGF and differing

amounts of EGFR antibody which was added to the growth media simultaneously with the EGF. MDA-468 growth media contained no EGF. Growth media was changed after 48 h and the cells were counted after 4 days. At the end of the experimental growth period cells were harvested with trypsin-EDTA and counted using a Particle Data cell counter (Particle Data, Inc., Elmhurst, IL). Data is % control cell numbers (mean±SD). 96 IgM (●), 42 IgM(○), nonspecific IgM(Δ), 225 IgG(■), 108 IgG (□), non-specific IgG(▲). (see Figure 13).

B. 96 Colony Inhibition Assay of 184A1N4 Cells

[0063] 184A1N4-T cells were suspended in semisolid agar medium containing 0.4% Bacto-Agar (Difco, Detroit, MI), IMEM, 10% FBS and treatments. Cells were plated (10,000/dish) into triplicate 35 mm culture dishes containing 1 ml IMEM, 0.6% agar and 10% FBS. The dishes were incubated for 10-14 days at 37°C in 5% CO_2 in the presence of 20 nM aEGFR or 20 nM nonspecific antibodies and increasing concentrations of EGF. Data are mean (±SD) number of colonies greater than 60 um in size. A) IgG:225IgG(●), 108 IgG (○), non-specific IgG (Δ). B) IgM: 96 IgM(○), 42 IgM(●), nonspecific IgM(Δ). Cell colonies larger than 60 um in diameter were counted using a Bausch & Lomb colony counter (See Figure 15).

C. 96 Colony Inhibition Assay of MDA-468 Cells

[0064] MDA-468 cells were suspended in semisolid agar medium containing 0.4% Bacto-Agar (Difco, Detroit, MI) IMEM, 10% FBS and treatments. Cells were plated (10,000/dish) into triplicate 35 mm culture dishes containing 1 ml IMEM, 0.6% agar and 10% FBS. The dishes were incubated for 10-14 days at 37°C in 5% CO_2 in the presence of 20 nM aEGFR or 20 nM nonspecific antibodies and increasing concentrations of EGF. Data are mean (±SD) number of colonies greater than 60 um. A) IgG:225IgG(●), 108 IgG (Δ), non-specific IgG (▲), EGF alone (○) .. B) IgM: 96 IgM(Δ), 42 IgM(●), nonspecific IgM(▲) EGF alone (○). Cell colonies larger than 60 nm in diameter were counted using a Bausch & Lomb colony counter. (See Figure 16).

EXAMPLE IX

Utility 108 Monoclonal Antibody Administered with Doxorubicin

[0065] Monoclonal antibody 108 was injected to form a subcutaneous tumor. Four doses of .45 mg of 108 monoclonal antibody and 37.5 ug of doxorubicin (adriamycin) were given 24 h after the tumor injection and repeated 3 times at 3-4 day intervals. The volume of the tumor was compared to the controls: phosphate buffered saline, antibody alone or drug alone. (See Figure 9).

Utility of 108 Monoclonal Antibody Administered with Cisplatin

[0066]

a) A single treatment comprising 1.8 mg 108 monoclonal antibody and 100 ug cisplatin was administered twenty-four hours after the subcutaneous tumor inoculation with $2X10^6$ KB cells. The results are presented in Figure 10.

b) A single treatment comprising 1.9 mg 108 monoclonal antibody and .1 ug cisplatin were injected intravenously each in a separate needle 20 h after the tumor transplantation. The combined treatment was significantly better than each of the treatments alone ($P < 0.02$ by student-t-test, $p<0.007$ by Mann-Whitney analysis) (See Figure 11).

Claims

1. A therapeutic composition comprising:

   (a) a monoclonal antibody which inhibits the growth of human tumor cells by said antibody binding to the extra-cellular domain of the human EGF receptors of said tumor cells in an antigen-antibody complex, said tumor cells being **characterized by** their expression of human EGF receptors and mitogenic stimulation by human EGF, and

   (b) an anti-neoplastic agent,

   wherein the antibody is not antibody 108 produced by hybridoma cell line ATCC HB 9764 or antibody 96 produced by hybridoma cell line ATCC HB 9763.

2. The therapeutic composition of claim 1 for separate administration of the components.

3. The therapeutic composition of claim 1 or 2 also comprising a pharmaceutically acceptable carrier.

4. The therapeutic composition of any one of claims 1 to 3 comprising an amount of the monoclonal antibody effective to inhibit the growth of human tumor cells that express human EGF receptors and that are mitogenically stimulated by human EGF.

5. The therapeutic composition of any one of claims 1 to 4 wherein the anti-neoplastic agent is doxorubicin or cisplatin.

6. Use of (a) a monoclonal antibody and (b) an anti-neoplastic agent, as defined in any one of claims 1 to 5, for the preparation of a therapeutic composi-

tion for treating cancer.

7. A process for the preparation of the therapeutic composition of any one of claims 1 to 5 which comprises combining the monoclonal antibody and the anti-neoplastic agent.

Patentansprüche

1. Therapeutische Zusammensetzung umfassend:

   (a) einen monoclonalen Antikörper, der das Wachstum menschlicher Tumorzellen durch Bindung des Antikörpers an die extrazelluläre Domäne der menschlichen EGF-Rezeptoren der Tumorzellen in Form eines Antigen-Antikörper-Komplexes hemmt, wobei die Tumorzellen durch Expression menschlicher EGF-Rezeptoren und mitogene Stimulation durch menschliches EGF gekennzeichnet sind, und
   (b) einen anti-neoplastischen Wirkstoff, wobei der Antikörper nicht der von der Hybridomzellinie ATCC HB 9764 produzierte Antikörper 108 oder der von der Hybridomzellinie ATCC HB 9763 produzierte Antikörper 96 ist.

2. Therapeutische Zusammensetzung nach Anspruch 1 für die getrennte Verabreichung der Komponenten.

3. Therapeutische Zusammensetzung nach Anspruch 1 oder 2, die ferner einen pharmazeutisch verträglichen Träger umfaßt.

4. Therapeutische Zusammensetzung nach einem der Ansprüche 1 bis 3, umfassend eine Menge des monoclonalen Antikörpers, die das Wachstum menschlicher Tumorzellen wirksam inhibiert, welche menschliche EGF-Rezeptoren exprimieren und durch menschlichen EGF mitogen stimuliert werden.

5. Therapeutische Zusammensetzung nach einem der Ansprüche 1 bis 4, wobei der anti-neoplastische Wirkstoff Doxorubicin oder Cisplatin ist.

6. Verwendung (a) eines monoclonalen Antikörpers und (b) eines anti-neoplastischen Wirkstoffs gemäß der Definition in einem der Ansprüche 1 bis 5 für die Herstellung einer therapeutischen Zusammensetzung zur Behandlung von Krebs.

7. Verfahren zur Herstellung der therapeutischen Zusammensetzung nach einem der Ansprüche 1 bis 5, wobei man den monoclonalen Antikörper und den anti-neoplastischen Wirkstoff miteinander kombiniert.

Revendications

1. Composition thérapeutique comprenant :

   (a) un anticorps monoclonal qui inhibe la croissance des cellules tumorales humaines par le fait que ledit anticorps se lie au domaine extracellulaire des récepteurs de EGF humain desdites cellules tumorales en un complexe antigène-anticorps, lesdites cellules tumorales étant **caractérisées par** leur expression de récepteurs de EGF humain et leur stimulation mitogène par EGF humain, et
   (b) un agent antinéoplasique,

   où l'anticorps n'est pas l'anticorps 108 produit par la lignée cellulaire d'hybridome ATCC HB 9764 ou l'anticorps 96 produit par la lignée cellulaire d'hybridome ATCC HB 9763.

2. Composition thérapeutique selon la revendication 1 pour l'administration séparée des composants.

3. Composition thérapeutique selon la revendication 1 ou 2 comprenant aussi un support pharmaceutiquement acceptable.

4. Composition thérapeutique selon l'une quelconque des revendications 1 à 3 comprenant une quantité de l'anticorps monoclonal efficace pour inhiber la croissance de cellules tumorales humaines qui expriment des récepteurs de EGF humain et qui sont stimulées de manière mitogène par EGF humain.

5. Composition thérapeutique selon l'une quelconque des revendications 1 à 4, où l'agent antinéoplasique est la doxorubicine ou le cisplatine.

6. Utilisation de (a) un anticorps monoclonal et (b) un agent antinéoplasique, comme défini dans l'une quelconque des revendications 1 à 5, pour la préparation d'une composition thérapeutique pour traiter le cancer.

7. Procédé de préparation de la composition thérapeutique selon l'une quelconque des revendications 1 à 5 qui comprend la combinaison de l'anticorps monoclonal et de l'agent antinéoplasique.

FIG. 1

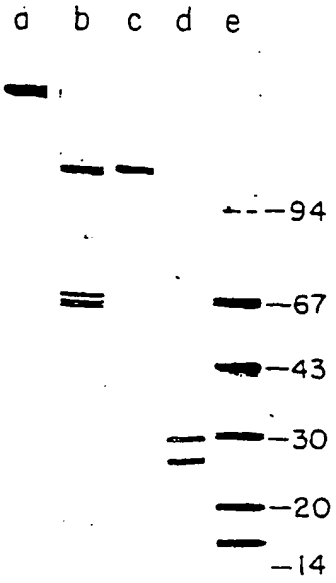


FIG. 3

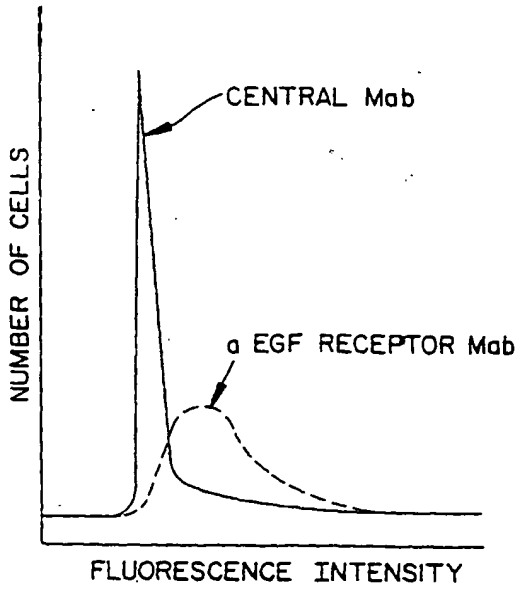


FIG. 2

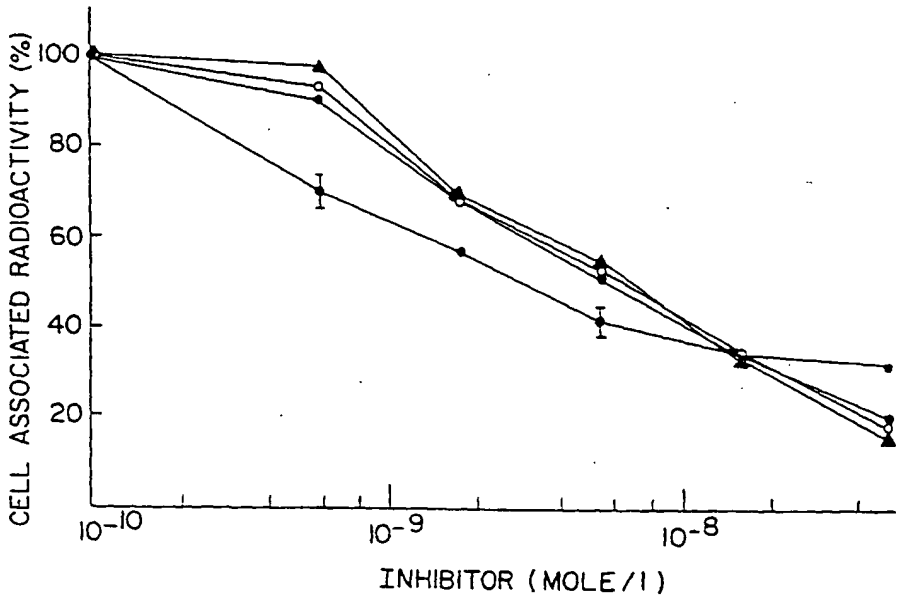
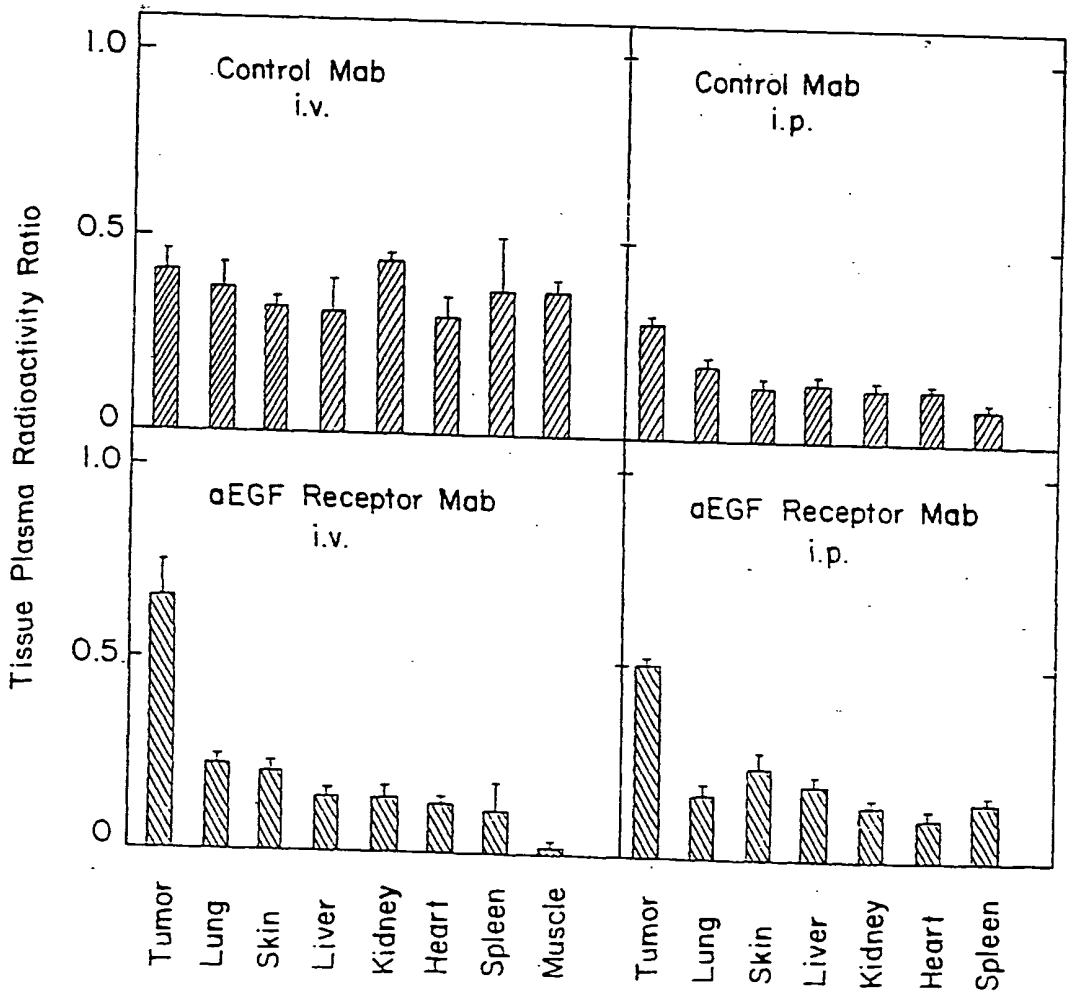


FIG.4


Tissue Plasma Radioactivity Ratio
1.0
0.5
0
Control Mab
i.v.
Control Mab
i.p.
αEGF Receptor Mab
i.v.
αEGF Receptor Mab
i.p.
Tumor
Lung
Skin
Liver
Kidney
Heart
Spleen
Muscle

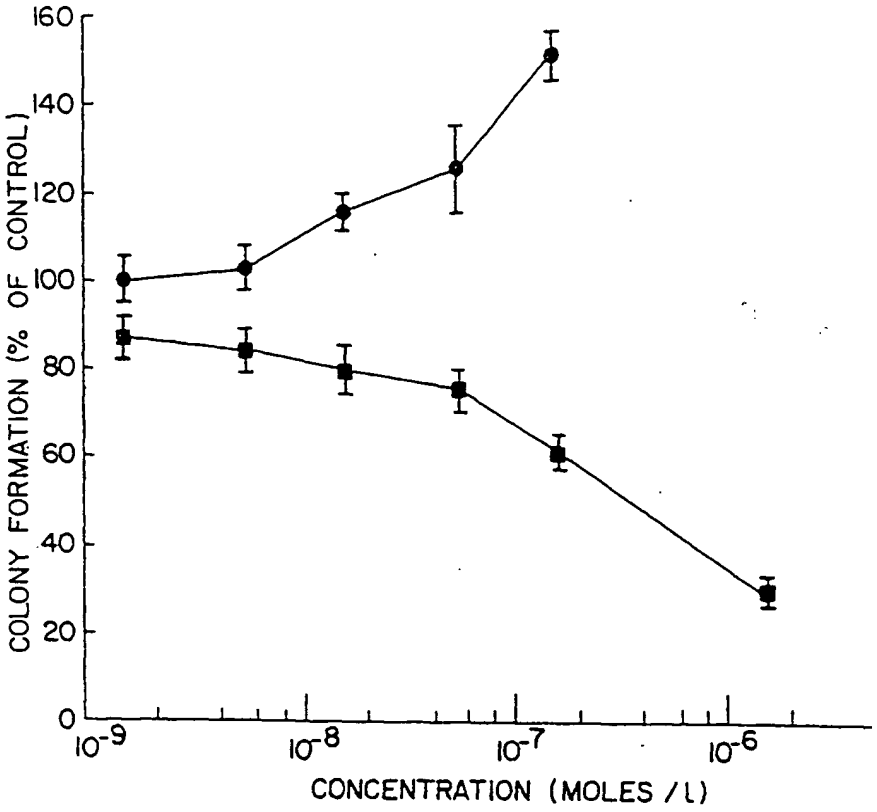

160
140
120
100
80
60
40
20
0
COLONY FORMATION (% OF CONTROL)
10^{-9}
10^{-8}
10^{-7}
10^{-6}
CONCENTRATION (MOLES / l)

FIG. 5

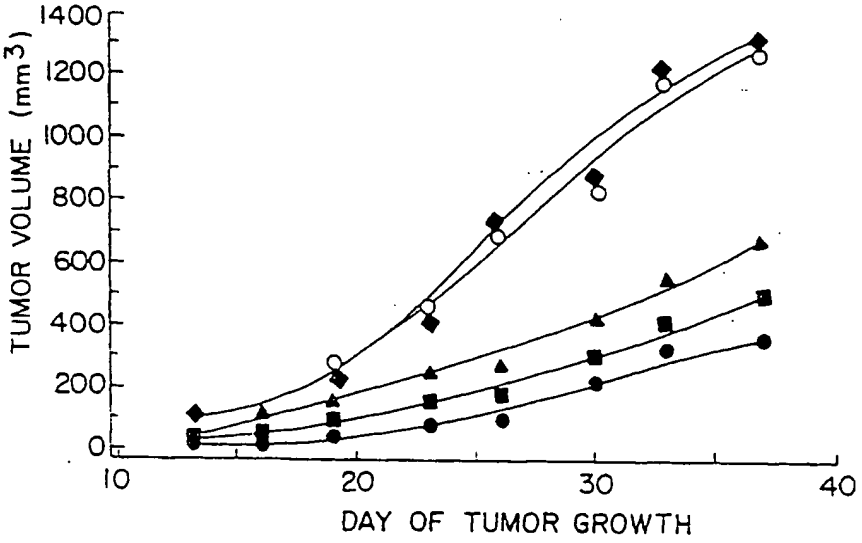

1400
1200
1000
800
600
400
200
0
TUMOR VOLUME (mm^3)
10
20
30
40
DAY OF TUMOR GROWTH

FIG. 6

FIG. 7

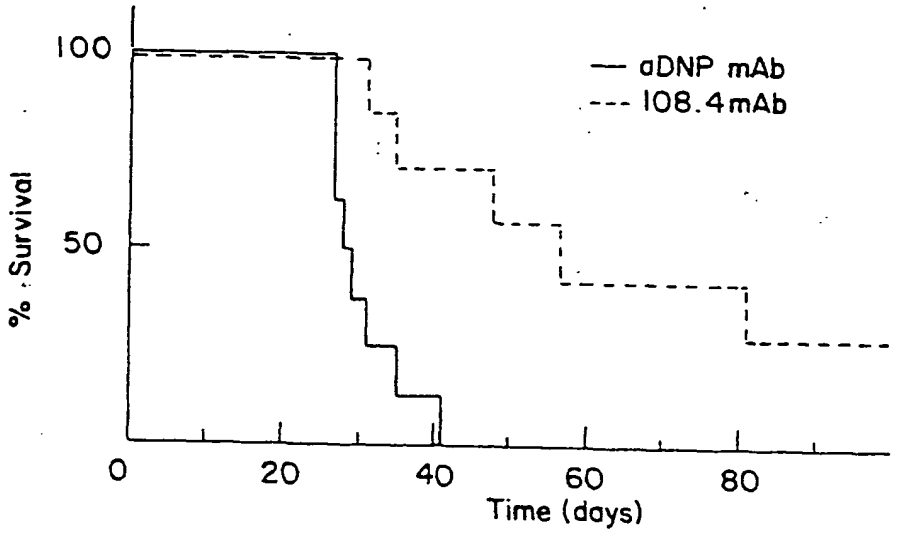

100
50
% Survival
0
20
40
60
80
Time (days)
— αDNP mAb
--- 108.4mAb

FIG. 9

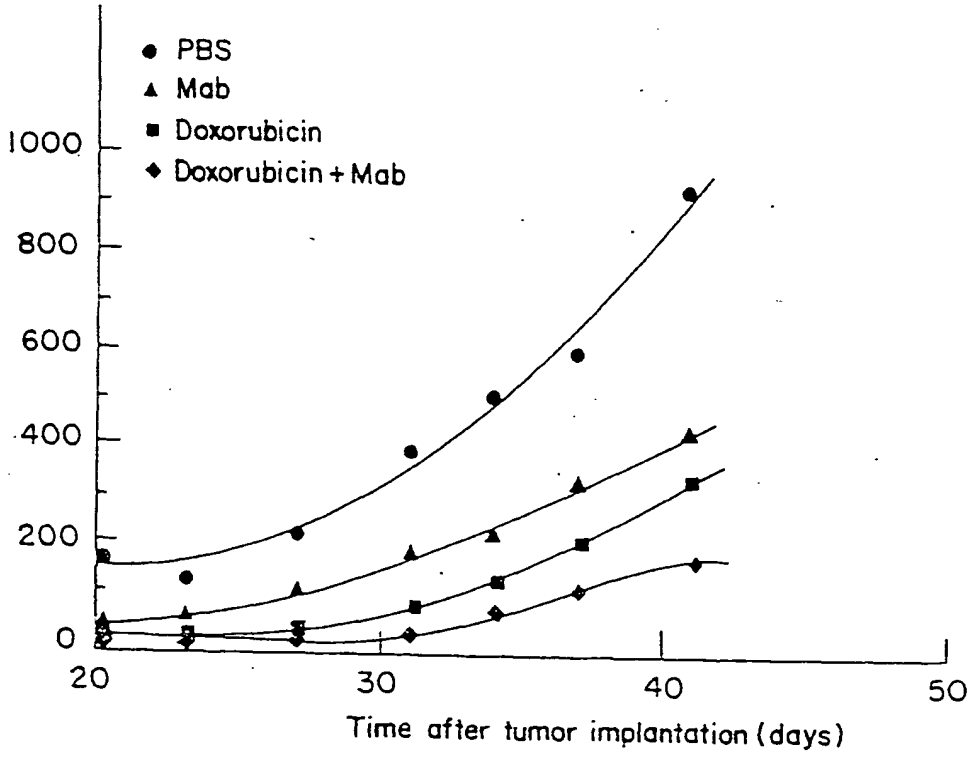
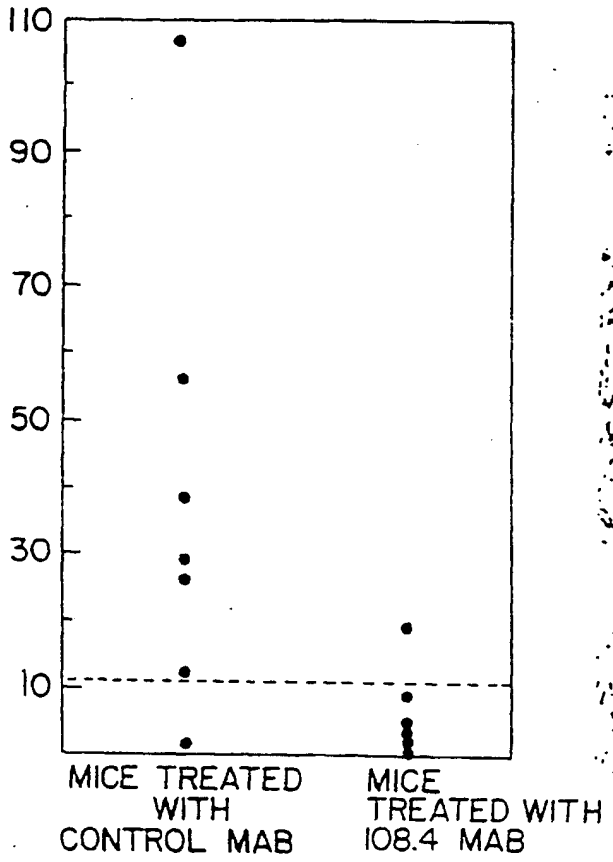
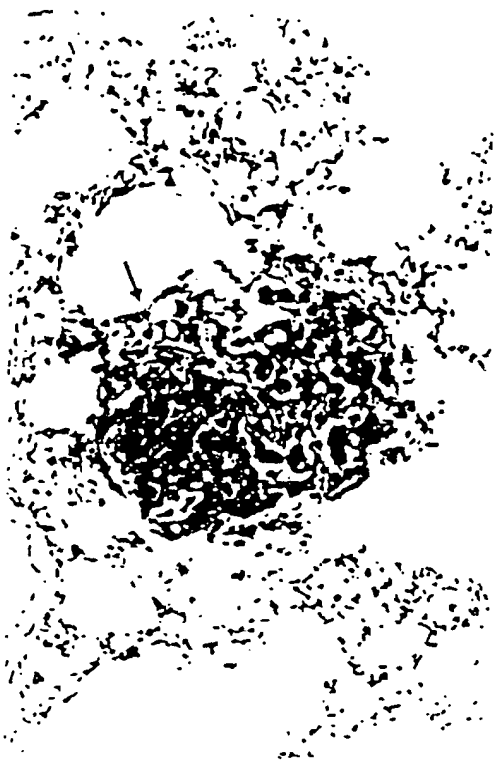

● PBS
▲ Mab
■ Doxorubicin
◆ Doxorubicin + Mab
1000
800
600
400
200
0
20
30
40
50
Time after tumor implantation (days)

FIG. 8





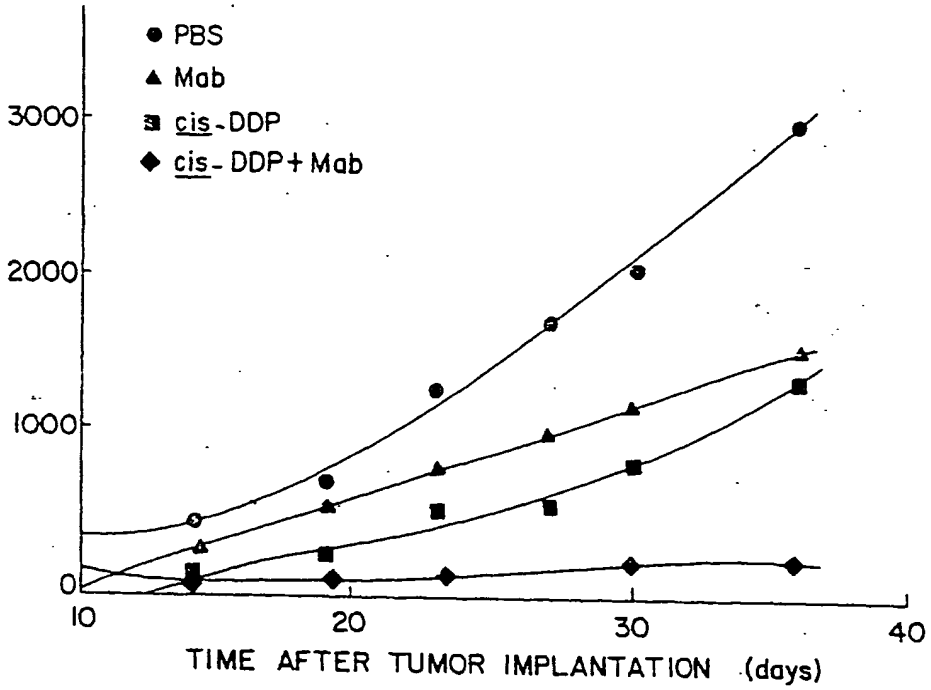

PBS
Mab
cis-DDP
cis-DDP+Mab
3000
2000
1000
0
10
20
30
40
TIME AFTER TUMOR IMPLANTATION (days)

FIG. 10

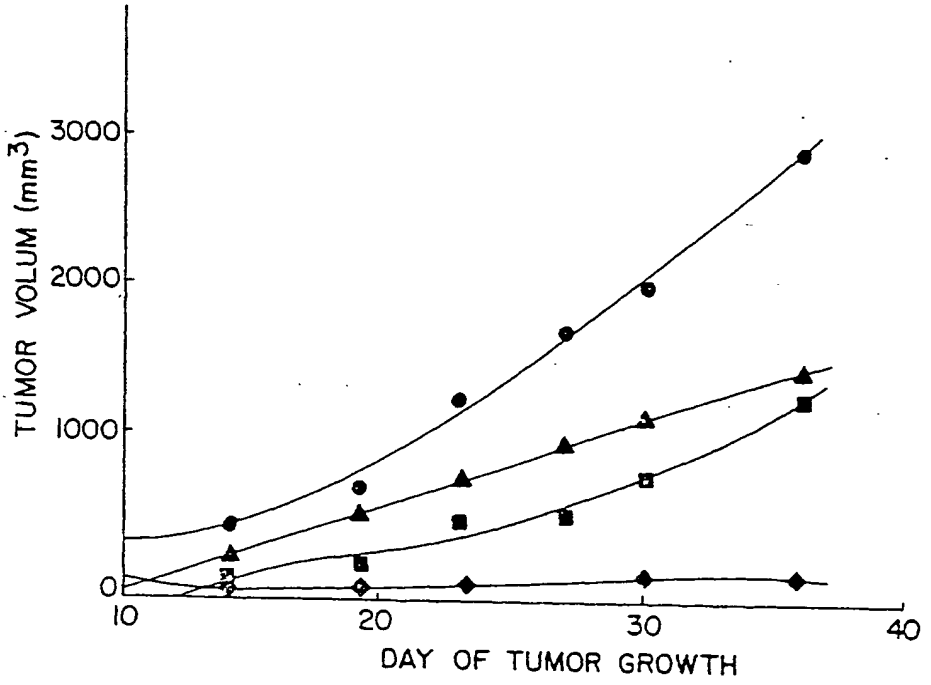

TUMOR VOLUM (mm3)
3000
2000
1000
0
10
20
30
40
DAY OF TUMOR GROWTH

FIG. 11

FIG. 12

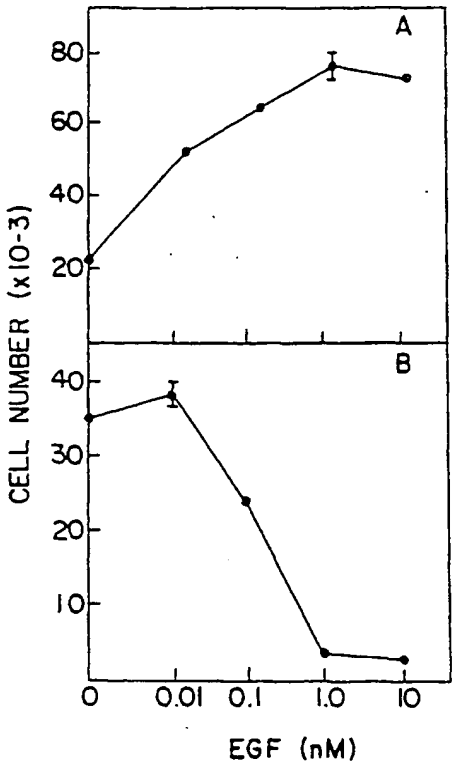
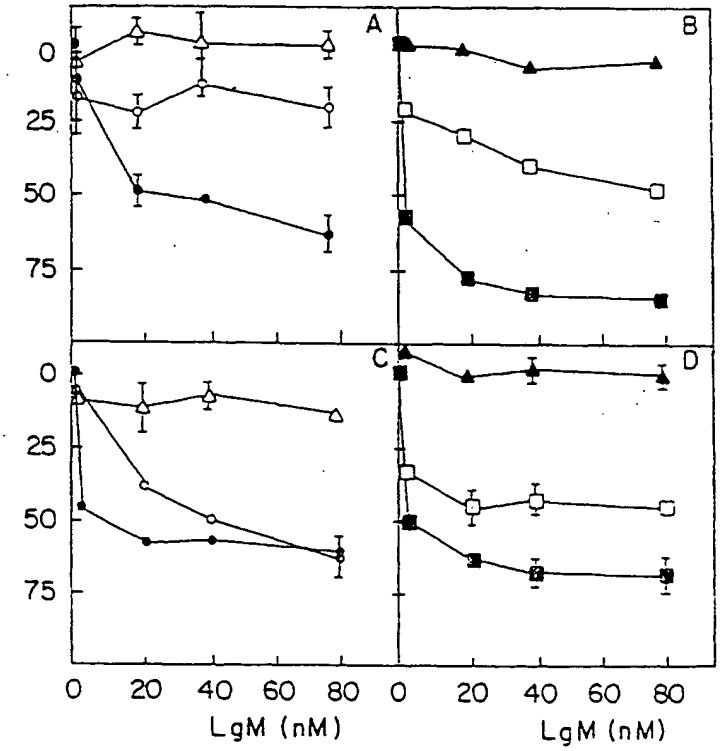

A
B
80
60
40
20
40
30
20
10
CELL NUMBER (x10-3)
0
0.01
0.1
1.0
10
EGF (nM)

FIG. 13


A
B
C
D
0
25
50
75
0
20
40
60
80
LgM (nM)
LgM (nM)

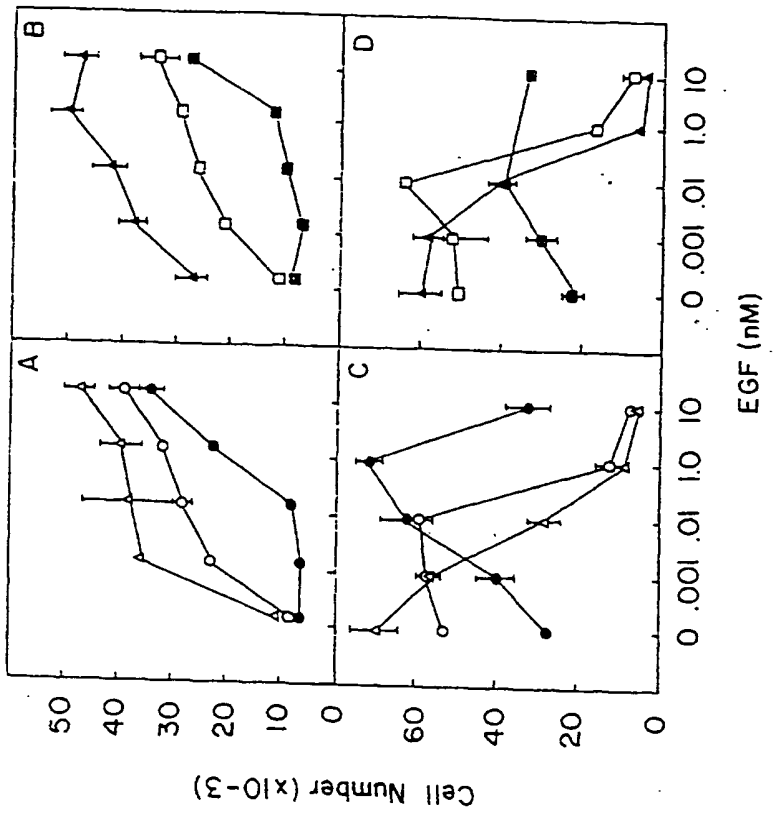

A
B
C
D
Cell Number (x10-3)
EGF (nM)
0 .0001 .001 .01 .1 1.0 10
0 10 20 30 40 50
0 20 40 60

FIG. 14

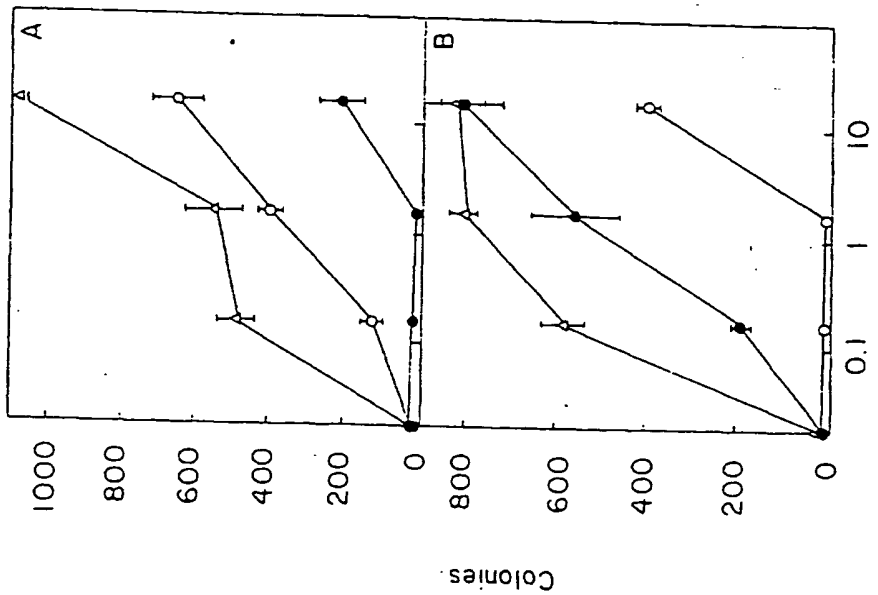

A
B
Colonies
EGF (nM)
0 200 400 600 800 1000
0 200 400 600 800
0.1 1 10

FIG. 15

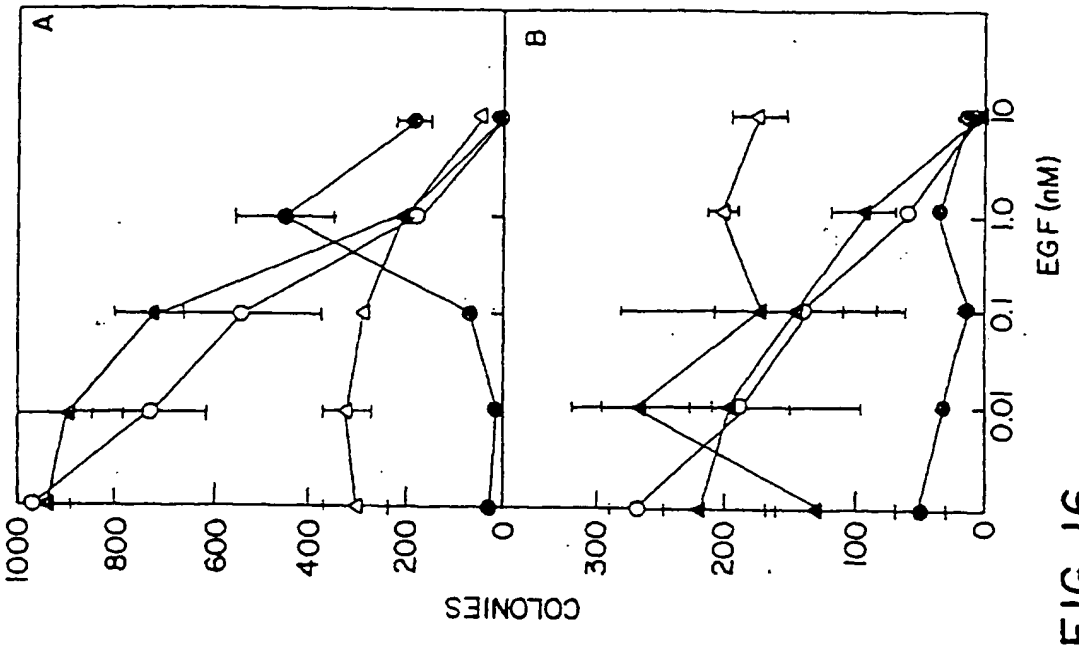

A
B
COLONIES
1000
800
600
400
200
0
300
200
100
0
0.01
0.1
1.0
10
EGF (nM)

FIG. 16

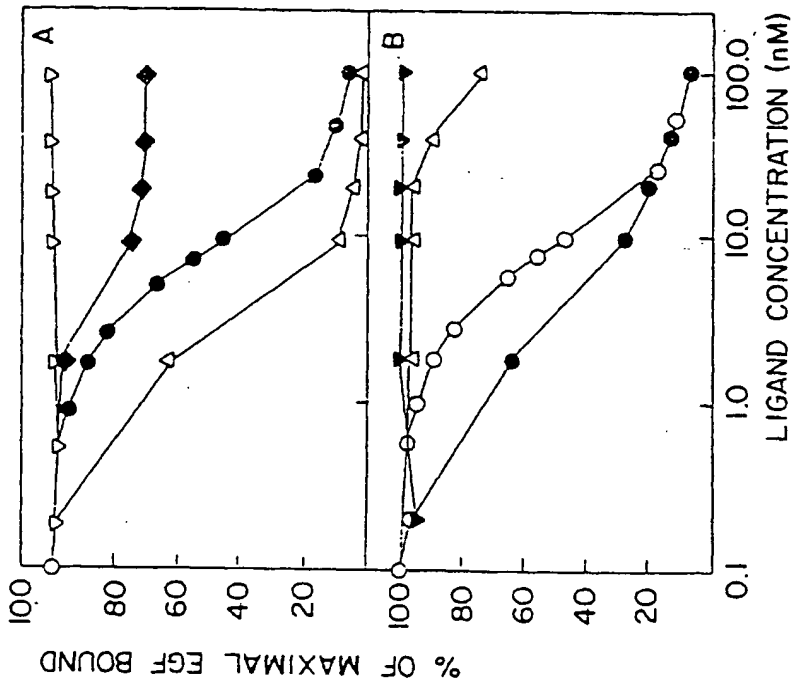

A
B
% OF MAXIMAL EGF BOUND
100
80
60
40
20
0.1
1.0
10.0
100.0
LIGAND CONCENTRATION (nM)

FIG. 17